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Alterations in spontaneous transmitter release by divalent cations after treatment of the neuromuscular junction with β-bungarotoxin

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Spontaneous transmitter release was studied at the frog sartorius neuromuscular junction in the presence of a variety of cations before and after treatment with the specific presynaptic neurotoxin, β-bungarotoxin (β-BuTX). Treatment with β-BuTX produced a maintained increase in spontaneous release, as indicated by the miniature end-plate potential (m.e.p.p.) frequency. It was demonstrated that the m.e.p.p. frequency remained dependent on the extracellular calcium concentration.

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20. ABSTRACT (continued)

A 30 mM increase in extracellular sodium chloride produced a reversible increase in frequency only after β-BuTX treatment, indicating that β-BuTX had increased the permeability of the presynaptic terminal. Furthermore, several divalent cations other than calcium were shown to either maintain or greatly increase the m.e.p.p. frequency after β-BuTX treatment (before toxin treatment replacement of calcium by these divalent cations produced only small changes in frequency). The relative effectiveness of the divalent cations tested in increasing spontaneous transmitter release after toxin treatment was $Co^{2+} \simeq Ni^{2} > Mg^{2+} > Ca^{2+} \simeq Sr^{2+} > Mn^{2+}$. The effect of cobalt, which increased the m.e.p.p. frequency 6.5 times after toxin treatment, was studied in detail.

It is proposed that β-BuTX, through its phospholipase activity, increases the ionic permeability of the terminal membrane and allows access to intracellular sites of relatively impermeant cations. This allowed us to demonstrate that several divalent cations other than calcium can influence transmitter release either directly at release sites or by altering internal calcium buffering.

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Alterations in Spontaneous Transmitter Release by Divalent Cations After Treatment of the Neuromuscular Junction with β-Bungarotoxin

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KEY WORDS: neuromuscular junction; transmitter release; β-bungarotoxin; calcium; divalent cations.

SUMMARY

1. Spontaneous transmitter release was studied at the frog sartorius neuromuscular junction in the presence of a variety of cations before and after treatment with the specific presynaptic neurotoxin, β-bungarotoxin (β-BuTX).

2. Treatment with β-BuTX produced a maintained increase in spontaneous release, as indicated by the miniature end-plate potential (m.e.p.p.) frequency. It was demonstrated that the m.e.p.p. frequency remained dependent on the extracellular calcium concentration.

3. A 30 m*M* increase in extracellular sodium chloride produced a reversible increase in frequency only after β-BuTX treatment, indicating that β-BuTX had increased the permeability of the presynaptic terminal.

4. Furthermore, several divalent cations other than calcium were shown to either maintain or greatly increase the m.e.p.p. frequency after β-BuTX treatment (before toxin treatment replacement of calcium by these divalent cations produced only small changes in frequency). The relative effectiveness of the divalent cations tested in increasing spontaneous transmitter release after toxin treatment was $Co^{2+} \simeq Ni^{2+} > Mg^{2+} > Ca^{2+} \simeq Sr^{2+} > Mn^{2+}$. The effect of cobalt, which increased the m.e.p.p. frequency 6.5 times after toxin treatment, was studied in detail.

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5. It is proposed that β-BuTX, through its phospholipase activity, increases the ionic permeability of the terminal membrane and allows access to intracellular sites of relatively impermeant cations. This allowed us to demonstrate that several divalent cations other than calcium can influence transmitter release either directly at release sites or by altering internal calcium buffering.

INTRODUCTION

A rise in intracellular calcium is believed to be a necessary step in neurosecretion and transmitter release. However, the mechanism by which Ca mediates transmitter release is uncertain. The role of Ca in this process has been studied by investigating the ability of other divalent cations to also support release. For example, strontium has been shown to replace Ca in both evoked and spontaneous release (Dodge *et al.*, 1969; Meiri and Rahamimoff, 1971; Miledi, 1966). It appears, thus far, to be the only divalent cation other than calcium which supports evoked release. By comparison, other divalent cations (e.g., Ba, Co, Mg, Mn, and Ni) do not support evoked release but can affect spontaneous release rates (Balnave and Gage, 1973; Hurlbut *et al.*, 1971; Kita and Van der Kloot, 1973, 1976; Misler and Hurlbut, 1979; Silinsky, 1977; Weakly, 1973). The ability of these other divalent cations to support spontaneous release is revealed only by conditions which most likely increase their access to the intracellular space, e.g., tetanic nerve stimulation (Hurlbut *et al.*, 1971; Kita and Van der Kloot, 1973), potassium depolarization (Kita and Van der Kloot, 1973), the presence of the calcium ionophore X537A (Kita and Van der Kloot, 1976), and black widow spider venom (Misler and Hurlbut, 1979). The inability of *some* of these ions to support evoked release can be explained by their inabiltiy to enter the cell through voltage-dependent calcium channels (Hagiwara *et al.*, 1974). An exception is Ba, which enters channels and allows release. Therefore, access to intracellular sites by divalent cations may be the primary limiting factor in their observed capacity to support release of transmitter.

Here, we report evidence that the specific presynaptic neurotoxin, β-bungarotoxin, (β-BuTX) can be used to increase access for these ions to the intracellular space. We find that the divalent ions Co^{2+}, Mg^{2+}, Ni^{2+}, and Sr^{2+} support spontaneous transmitter release after β-BuTX treatment. However, the different time course of the effects of these divalent cations suggest that they may not all be acting through a single mechanism.

MATERIALS AND METHODS

Experiments were performed on the sartorius neuromuscular junction of the frog, *Rana pipiens*. Approximately 1 cm of the sciatic nerve was retained and tied off in each preparation. The control Ringer solution (115 m*M* NaCl, 2.5 m*M* KCl, 1.8 m*M* $CaCl_2$, 3.0 m*M* Hepes buffer, pH 7.0) contained neostigmine bromide (3 μ*M*) (Sigma) and tetrodotoxin (1 μ*M*) (Sigma) to enhance the miniature end-plate potential (m.e.p.p.) amplitude and to inhibit the spontaneous muscle twitching that occurred after β-BuTX treatment and interfered with long-term microelectrode

penetration of single muscle fibers. Neostigmine and tetrodotoxin, which were present in all solutions, did not influence the β-BuTX-induced effects described here. Frog muscles were superfused in a water-jacketed plexiglass chamber at temperatures of 18–21°C. The muscle was pinned on a Sylgard resin surface. The placement of microelectrodes was under direct visual control using a compound microscope (115×). The m.e.p.p. frequency and resting membrane potential were monitored by conventional microelectrode techniques (Livengood *et al.*, 1978). Electrophysiological signals were digitized and analyzed by an on-line computer (PDP-11/34, DEC, Maynard, MA).

β-Bungarotoxin (22,000-dalton protein) was isolated by passage of the crude venom (Miami Serpentarium, Miami, FL) through two ion-exchange columns (C-50 and G-75, Sephandex) (Tobias *et al.*, 1978). Commercially purified β-BuTX (Boehringer Mannheim, Indianapolis, IN) gave similar results. The procedure for β-BuTX treatment was as follows: the neuromuscular preparation was exposed to a β-BuTX concentration of 0.1–2.0 μg/ml. During this time perfusion was stopped and the chamber fluid was mixed by a push–pull syringe system immediately after the addition of a concentrated aliquot of β-BuTX. Ten minutes later, the chamber fluid was mixed again. Twenty minutes after the beginning of β-BuTX exposure, the chamber was rapidly flushed with β-BuTX free Ringer solution. The β-BuTX concentration was varied to produce comparable increases in m.e.p.p. frequency in different preparations. This variability in β-BuTX potency reflected differences in β-BuTX batches, sources, and variability among end plates. Divalent cations were added as their chloride salts.

RESULTS

β-Bungarotoxin has been shown previously to influence spontaneous and evoked release at the neuromuscular junction. Spontaneous release initially increased and then began to fall within 2 hr, eventually leading to transmission failure after exposure to β-BuTX (Lee and Chang, 1966; Oberg and Kelly, 1976; Kelly and Brown, 1974; Abe *et al.*, 1976). Several groups have suggested the importance of the phospholipase A_2 activity of β-BuTX as the basis for its action (Abe and Miledi, 1978; Livengood *et al.*, 1978; Strong *et al.*, 1976; Wernicke *et al.*, 1975). More detailed studies have linked the changes in transmitter release and the cessation of transmission with the phospholipase activity (Abe and Miledi, 1978; Chang *et al.*, 1973; Howard and Truog, 1977; Kelly *et al.*, 1976; Livengood *et al.*, 1978). These changes in synaptic transmission can be explained by an increase in ionic permeability of the presynaptic terminal membrane. The eventual block of transmission, with relatively high β-BuTX concentrations, can be attributed to the breakdown of the terminal membrane (Abe *et al.*, 1976; Chen and Lee, 1970; Strong *et al.*, 1977). Therefore a relatively low concentration (0.1–2 μg/ml) of β-BuTX was used to increase the permeability of the presynaptic terminal and maintain an increased spontaneous release of transmitter without a full block of transmission.

The time course of the m.e.p.p. frequency changes during and after exposure of a muscle to β-BuTX is illustrated in Fig. 1. During the 20-min period of exposure to toxin, the m.e.p.p. frequency decreased and then increased to 5–10 times the pretoxin

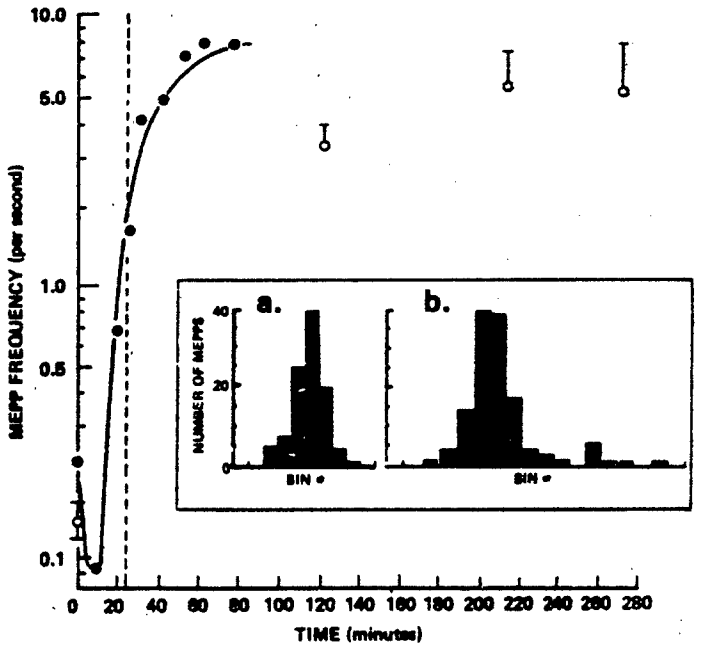

Fig. 1. Change in m.e.p.p. frequency after β-BuTX treatment. At time zero, the neuromuscular preparation was exposed to 0.1 μg/ml β-BuTX in control Ringer. After approximately 20 min, at the vertical dashed line, the preparation was washed with β-BuTX-free control Ringer. The filled circles are frequency measurements from a single end plate followed until the penetration was lost. The open circles and bars represent the mean and SE of m.e.p.p. frequencies from at least 10 end plates from the same muscle. The m.e.p.p. frequency first decreased and then increased rapidly to a steady level 2 hr after toxin exposure. The inset illustrates the amplitude histograms of m.e.p.p.s before (a) and after (b) exposure to toxin. The bin width is 160 μV and the base bin is 0–160 μV. The model amplitude was unchanged after toxin treatment, however, events that were twice the modal amplitude were present after toxin treatment. The bins not shown in inset (a) were empty.

rate. The m.e.p.p. frequency plateaued and remained elevated for up to 6 hr. All results reported as "after β-BuTX pretreatment" in this and subsequent experiments were recorded after the muscle was treated with β-BuTX for 20 min and then washed with toxin-free control Ringer for at least 2 hr. Previous experiments by others (Kelly and Brown, 1974; Oberg and Kelly, 1976) using a higher concentration of β-BuTX, e.g., 20 μg/ml, showed spontaneous release peaking within 60 min and then steadily decreasing thereafter. The mode of the m.e.p.p. amplitude frequency distribution was not significantly altered by the toxin treatment (inset, Fig. 1). However, larger m.e.p.p.s of twice the modal amplitude were also present. When commercially purified β-BuTX was used, there was a rapid decrease in the m.e.p.p. amplitude; however, after 2 hr of washing with β-BuTX free solution, the m.e.p.p. amplitude recovered to the control level. This effect could be explained by a contaminant that had a reversible postsynap-

tic action (MacDermot *et al.*, 1978). A majority of these results was obtained using commercially purified toxin.

The effect of β-BuTX treatment on the functional relationship between m.e.p.p. frequency and extracellular calcium concentration was examined between 1.0 and 16 m*M* extracellular calcium (Fig. 2). Without toxin treatment, m.e.p.p. frequency was proportional to extracellular calcium concentration; the slope of the log–log plot was 1. However, after toxin pretreatment, the slope decreased to 0.55 (mean = 0.37 ± 0.067, $N = 3$). In contrast, Oberg and Kelly (1976) found that the functional dependence of m.e.p.p. frequency on external Ca was not affected by 20-μg/ml β-BuTX treatment in the rat phrenic nerve–diaphragm preparation. The different result might be due to their use of a higher toxin concentration or a mammalian preparation. The cause of the decreased slope after toxin treatment is not known; however, the fact that the m.e.p.p. frequency remained Ca dependent allowed us to investigate the effect of other divalent cations on the m.e.p.p. frequency after toxin treatment. The calcium-dependent increases in the m.e.p.p. frequency following toxin treatment were reversible, so that

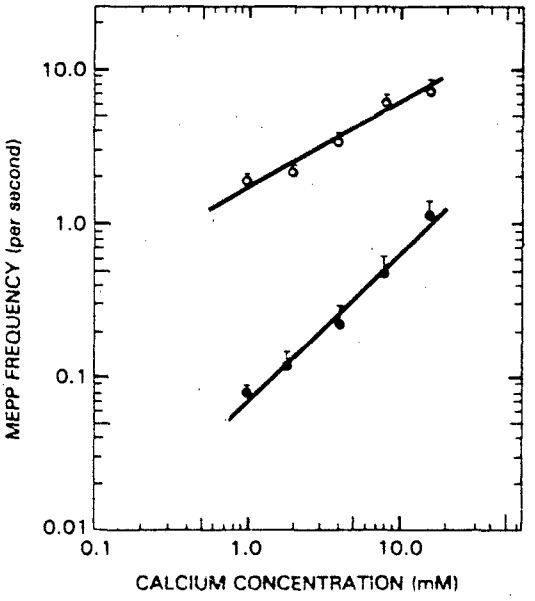

Fig. 2. The relationship between m.e.p.p. frequency and calcium concentration in β-BuTX-treated and untreated muscles. The m.e.p.p. frequency of β-BuTX-treated, 1 μg/ml (○), and untreated (●) muscles increased with increasing calcium concentrations (1.0, 1.8, 4.0, 8.0, and 16 m*M*). The relationship under the two conditions varied, showing that m.e.p.p. frequencies of the toxin-treated preparations increased at a slower rate than in nontreated muscles. The data points are averages of 10 fibers under control conditions and 3 fibers after toxin treatment. Both coordinates are logarithmic. Standard error bars are indicated.

the increases could not have resulted from further phospholipase activation (Howard and Truog, 1977; Strong *et al.*, 1976; Tobias *et al.*, 1978).

Increasing the intracellular sodium ion concentration modulates transmitter release presumably by altering internal calcium concentrations (Baker and Crawford, 1975; Birks and Cohen, 1968; Charlton and Atwood, 1977; Rahamimoff *et al.*, 1978a, b). In untreated muscle, when the sodium chloride concentration of the Ringer solution was raised by 30 m*M*, there was no change in frequency (Fig. 3). However, after toxin treatment elevating the sodium chloride increased the frequency by a factor of 10 (Fig. 3), and the effect was reversible. After washout with normal Ringer, the frequency declined to one-half the maximum frequency within 4 min and returned to the control level within 30 min.

External calcium was not necessary to elicit the Na effect. When calcium was replaced by 2 m*M* manganese and the sodium concentration raised by 30 m*M*, the m.e.p.p. frequency again increased but only after toxin treatment. As will be seen later, 2 m*M* manganese did not produce an increase in m.e.p.p. frequency after toxin treatment. This increase in frequency was due most likely to the increase in sodium influx due to phospholipase treatment and a release of calcium from internal stores.

Previous work has shown that most divalent cations cannot support evoked transmitter release (Del Castillo and Engbaek, 1954; Kita and Van der Kloot, 1973; Meiri and Rahamimoff, 1971; Miledi, 1966; Silinsky, 1977; Weakly, 1973) probably because of the low permeability to these ions of the voltage-dependent calcium channels in the presynaptic terminal. However, if as suggested above, β-bungarotoxin does increase the permeability of the terminal membrane, toxin treatment might allow us to compare more directly the ability of various divalent cations to produce

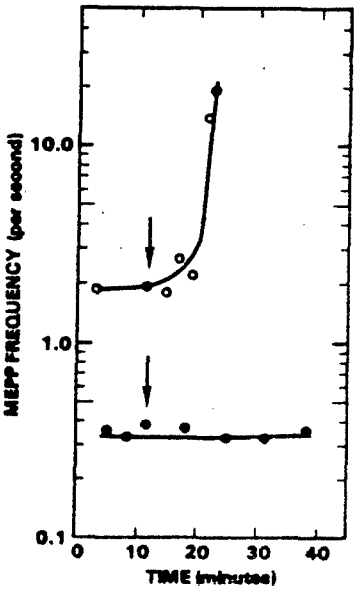

Fig. 3. The effect of elevated NaCl on m.e.p.p. frequency. The arrows point to the time at which the bathing solution was switched from the control Ringer to a solution that contained 30 m*M* more NaCl. The m.e.p.p. frequencies recorded from a junction of an untreated muscle were unchanged after exposure to the elevated NaCl Ringer as illustrated by the filled circles. However, after β-BuTX treatment (1 μg/ml), the m.e.p.p. frequency increased rapidly 10 min after switching to the test solution as illustrated by the open circles.

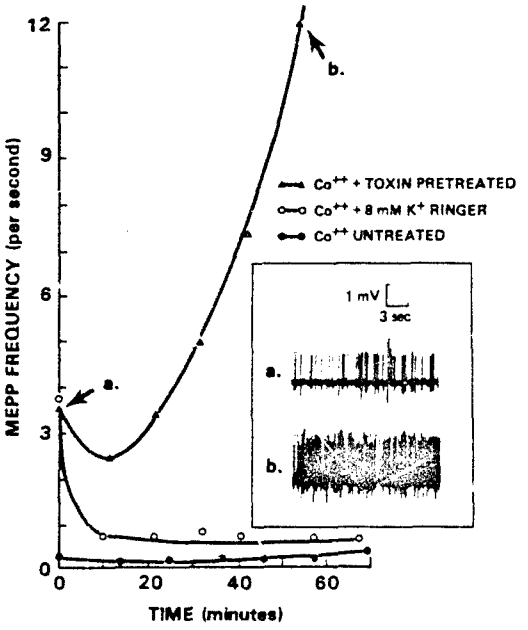

Fig. 4. The effects of cobalt chloride (2 m*M*) on m.e.p.p. frequency under various experimental conditions. Cobalt had a negligible effect on the m.e.p.p. frequency of a muscle which had not been exposed to β-BuTX (●). After toxin treatment (0.2 μg/ml), cobalt first decreased the m.e.p.p. frequency and then increased the frequency to more than three times that under post-toxin conditions in 1.8 m*M* calcium Ringer (Δ). By comparison, cobalt decreased the m.e.p.p. frequency in a fiber from a non-toxin-treated muscle exposed to 8 m*M* potassium chloride during the same time period (○). Potassium, 8 m*M*, elevated the m.e.p.p. frequency to approximately that of toxin-treated muscles in control Ringer. The recording chamber was perfused with a cobalt chloride Ringer solution starting at time zero in all cases. The inset illustrates chart recordings of miniature end-plate potentials of the toxin-treated fiber before (a) and after (b) switching to 2 m*M* cobalt chloride Ringer.

transmitter release by circumventing the specificity of the channel. The effects of cobalt, magnesium, manganese, nickel, and strontium were also examined before and after toxin treatment. Of these, the effect of cobalt was studied in detail. Replacement of Ca by 2 m*M* cobalt chloride was without effect on the m.e.p.p. frequency in untreated muscle (filled circles, Fig. 4). However, after toxin treatment, 2 m*M* cobalt increased the m.e.p.p. frequency by about 6.5 times, i.e., from 6.4 ± 1.0 to 42.0 ± 8.0 Hz (Table I). Maximal m.e.p.p. frequency was reached about 90 min after switching to the Co solution; the frequency then decayed to a plateau below control (0.65 ± 0.042 Hz, $N = 11$) after an additional 80 min.

Kita and van der Kloot (1973) observed changes in release rate in the presence of Co or Ni when the frog neuromuscular junction was tetanically stimulated or

Table I. Changes in M.e.p.p. Frequency Due to Divalent Cation Replacement of Calcium Before and After Toxin Treatment

Replacement ion	Conc. (m*M*)	Untreated (mean ± SE)	Toxin treated (mean ± SE)
Cobalt	2	0.23 ± 0.02 → 0.33 ± 0.04 (+43%)	6.40 ± 1.0 → 42.0 ± 8.0 (+556%)
Magnesium	2	0.50 ± 0.10 → 0.22 ± 0.04 (−56%)	5.64 ± 1.04 → 11.70 ± 2.09 (+107%)
	5	0.15 ± 0.02 → 0.07 ± 0.01 (−53%)	2.36 ± 0.52 → 11.16 ± 3.46 (+373%)
Manganese	2	0.20 ± 0.04 → 0.36 ± 0.08 (+80%)	4.0 ± 0.55 → 2.5 ± 0.4 (−38%)
Nickel	2	0.41 ± 0.07 → 0.28 ± 0.10[a] (−32%)	1.6 ± 0.4 → 9.4 ± 1.0 (+488%)
Strontium	2	0.26 ± 0.03 → 0.17 ± 0.02 (−35%)	5.20 ± 0.8 → 5.30 ± 1.0 (+2%)
	5	0.19 ± 0.02 → 0.17 ± 0.02 (−11%)	1.68 ± 0.39 → 3.35 ± 0.70 (+99%)

[a] $N = 3$; $N > 10$ in all other cases.

depolarized with potassium. However the effects of Co were not due to the depolarization of the presynaptic terminal membrane which may have been produced by toxin treatment. This was demonstrated by depolarizing untreated neuromuscular preparations with a Ringer solution containing 8 m*M* K^+, which increased the m.e.p.p. frequency to the same level exhibited by β-BuTX-treated muscles in control Ringer. As shown in Fig. 4, the exchange of 2 m*M* cobalt for normal calcium decreased the frequency without the subsequent potentiation which was characteristically seen after toxin pretreatment at a normal K^+ concentration. The decrease in frequency may be explained by the decrease in calcium influx by cobalt blockage of calcium channels. Therefore, it does not appear that a depolarization produced by toxin treatment simply opens channels permeable to cobalt or exposes sites for cobalt that are not open at a normal resting membrane potential.

The increase in frequency after the replacement of calcium with cobalt must be related to an action not related to depolarization by β-BuTX. This could simply reflect additional activation of toxin molecules by cobalt. This possibility was ruled out by investigating whether the toxin could produce its initial effects in the presence of cobalt alone. To do this, neuromuscular preparations were soaked in 2 m*M* cobalt chloride, calcium-free Ringer for 1 hr and then exposed to toxin in the usual manner in this medium (Fig. 5). No change in m.e.p.p. frequency was seen during exposure to the toxin. During the β-BuTX-washout period, there was a gradual increase in frequency, but it was not until the muscle was returned to control calcium Ringer that the frequency increased at a rapid rate. This result indicates that cobalt does not block the binding of β-BuTX significantly and does not effectively activate the phospholipase action. Only when calcium is present is the phospholipase activity evident (Abe *et al.*, 1976; Chang *et al.*, 1977; Howard and Truog, 1977; Strong *et al.*, 1976; Tobias *et al.*, 1978).

Thus far, it appeared that the increase in the potency of cobalt on release was due to an increase in the membrane permeability by β-BuTX. However, it was not clear

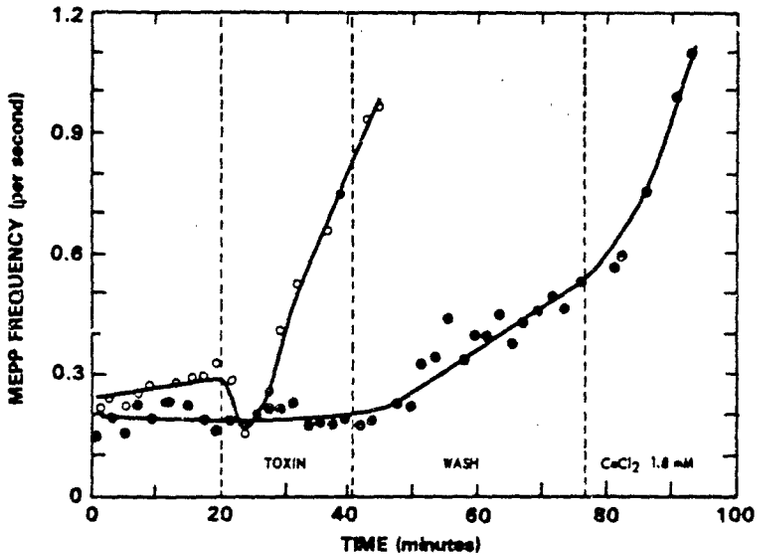

Fig. 5. The effect of β-BuTX on m.e.p.p. frequency in the presence of 2 m*M* cobalt chloride. A muscle was exposed to 2 m*M* cobalt Ringer for 1 hr before treatment with toxin (0.1 μg/ml) (●). No significant change in frequency was observed during the toxin exposure period. It was during this period in another muscle treated with toxin in control Ringer (○) that the m.e.p.p. frequency was potentiated. In the cobalt-exposed muscle fiber (●), after washing with β-BuTX-free, cobalt Ringer solution for 36 min, the replacement of cobalt by 1.8 m*M* calcium resulted in a rapid increase in m.e.p.p. frequency. This increase had the same rate course as the fiber treated with toxin (0.1 μg/ml) while bathed in control Ringer solution (1.8 m*M* calcium) (○).

how cobalt was potentiating the release of transmitter. With a simple increase in permeability, the reversibility of these effects might be rapid, recovering within minutes after returning to the control ionic environment. The sodium- and calcium-dependent increases in frequency were reversible in this way. However, this was not the case for Co. The exposure of a β-BuTX-treated terminal to Co increased the m.e.p.p. frequency to 8 Hz; the return to the control Ringer produced a further increase in m.e.p.p. frequency that was unaltered with a subsequent replacement of calcium by cobalt. In untreated neuromuscular junctions, at a higher concentration of cobalt (10 m*M*) the rise in the m.e.p.p. frequency was reversed for a period of 30 min before the frequency began to rise again, indicating both a direct effect that was reversible and a more long-term effect. Such data indicated that cobalt, in addition to having a direct effect on release, may be interfering with intracellular maintenance of calcium levels during steady calcium influx.

The possibility that cobalt may have an indirect effect on transmitter release are also further suggested by the latency of the calcium-induced m.e.p.p. increases (Fig. 6). When the extracellular calcium concentration was raised from 1.8 to 16 m*M*, the m.e.p.p. frequency of both treated and untreated junctions (untreated shown) rose rapidly to a plateau within a few minutes after changing solutions. However, after toxin treatment, the replacement of cobalt for calcium produced an increase after a

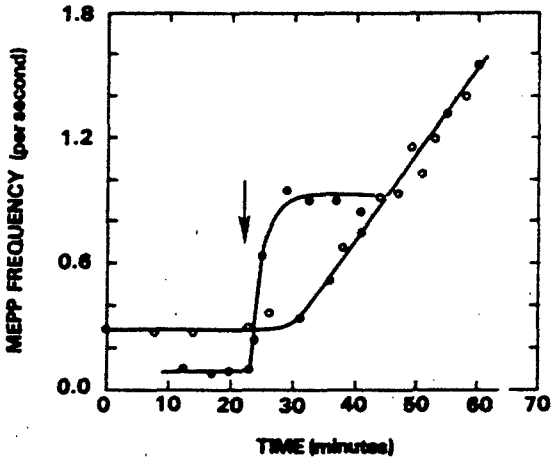

Fig. 6. The time course of the m.e.p.p. frequency increase in 16 m*M* calcium Ringer and 2 m*M* cobalt Ringer. The increased m.e.p.p. frequency after β-BuTX treatment (2 μg/ml) and exposure to 2 m*M* cobalt (O) was slow relative to the response of preparations that were untreated and exposed to 16 m*M* calcium (●). In addition, there was an early plateau of m.e.p.p. frequencies in muscles exposed to 16 m*M* calcium during the time that the frequency continued to increase in 2 m*M* cobalt. Solution change is indicated by the arrow.

delay of about 5 min. The frequency then rose at a slower rate and did not always reach a plateau during the ususal recording period of 1 hr. The delay of cobalt's effect may reflect a less direct site of action with respect to the site of transmitter release.

The effects of other divalent cations were also examined in β-BuTX-treated muscles (Table I). In all experiments, the divalent cations replaced calcium in the Ringer solution. The frequencies of m.e.p.p.s were recorded in single fibers during solution changes and when the frequency reached a steady state, several more end plates were sampled. When steady state was not reached as in some of the end plates exposed to cobalt and nickel, sampling was performed within a 1-hr period at which time a maximum frequency was achieved. Nickel chloride, 2 m*M*, produced an effect similar to that of cobalt, by increasing the frequency by 486% after toxin treatment, in comparison to a decrease of 32% before toxin treatment. Manganese chloride, 2 m*M*, however, produced a decrease of 38% in m.e.p.p. frequency after toxin treatment. Higher manganese concentrations were not used since they potentiated the m.e.p.p. frequency even without toxin treatment (data not shown; Balnave and Gage, 1973). Strontium chloride did not change the m.e.p.p. frequency before or after toxin treatment, which is in agreement with the present understanding that strontium can replace calcium in many functions. Raising strontium to 5 m*M* after toxin treatment produced an increase comparable to that seen with the same increase in calcium concentration. Magnesium chloride, 2 m*M*, increased the m.e.p.p. frequency by 107% after toxin treatment, compared to a decrease of 56% under control conditions. Increasing the magnesium concentration to 5 m*M* further increased the m.e.p.p.

frequency after toxin treatment. All of these changes were reversible except for those of nickel and cobalt, in which the m.e.p.p. frequency rose and then fell within the recording time in a similar manner. The relative effectiveness of the divalent cations in their ability to increase the m.e.p.p. frequency after toxin treatment was as follows: $Co^{2+} \simeq Ni^{2+} > Mg^{2+} > Ca^{2+} \simeq Sr^{2+} > Mn^{2+}$.

DISCUSSION

We have used a specific presynaptic neurotoxin, β-bungarotoxin, to modify the nerve terminal of the frog neuromuscular junction. The treatment with low concentrations of β-BuTX revealed the ability of several divalent cations to change the spontaneous release rate of quantal acetylcholine. The simplest interpretation of these results is that toxin treatment increases the permeability of the presynaptic membrane to cations that normally are considered impermeable, i.e., Co, Mg, Mn, and Ni. The phospholipase activity of β-BuTX could play a crucial role in increasing membrane permeability by phospholipid hydrolysis. As was shown in Ca^{2+}-loaded sarcoplasmic reticulum, β-BuTX increased the passive calcium efflux (Lau *et al.*, 1974). Abe *et al.* (1976) observed multiple firing in stimulated nerves of neuromuscular preparations that have been treated with 5 μg/ml toxin, indicating a possible increase in membrane permeability and depolarization. Other workers have shown electronmicrographs of presynaptic terminals of neuromuscular junctions after transmission failed due to β-BuTX treatment, and they demonstrated alterations of the plasma membrane and swelling of mitochondria (Abe *et al.*, 1976; Chang *et al.*, 1977 Ng and Howard, 1978), which can be a reflection of an increase in calcium influx and plasma membrane depolarization.

The similarity of the toxin-induced effects of increasing external Na reported here to manipulations of intracellular sodium reported elsewhere (Charlton and Atwood, 1977; Rahamimoff *et al.*, 1978a; Rahamimoff *et al.*, 1978b) further supports the conclusion of an increase in membrane permeability. In addition, the recovery from high sodium was complete and rapid, which suggested a rapid sodium dissociation and efflux. These results, however, do not rule out the possibility that sodium had either an effect on the external surface of the membrane that was enhanced by toxin treatment or a direct effect on release sites.

As expected from a β-BuTX-induced increase in membrane permeability, previously impermeant divalent cations increased spontaneous release. The relative potency of these cations at 2 m*M* concentrations on spontaneous transmitter release was $Co^{2+} \simeq Ni^{2+} > Mg^{2+} > Ca^{2+} \simeq Sr^{2+} > Mn^{2+}$ and could reflect the selectivity of either the toxin-induced increase in permeability or the sites of action of these cations. A further activation of the phospholipase by divalent cations after pretreatment does not appear to be significant in determining this relative potency in view of the demonstration that the divalent cations Mg, Mn, and Sr cannot support phospholipase activity (Chang *et al.*, 1977; Howard and Troug, 1977; Strong *et al.*, 1976). In addition, we have shown (Fig. 5) that cobalt also cannot readily support the enzyme activity.

It has been demonstrated by others that, under differing conditions, spontaneous

release increases in the presence of the cations studied here. Conditions included high extracellular concentrations of divalent cations (Kita and Van der Kloot, 1973; Kita *et al.*, 1981; Weakly, 1973), and neural tetanic stimulation or K depolarization (Hurlbut *et al.*, 1971; Kita and Van der Kloot, 1973; Kita *et al.*, 1981). Permeation presumably occurred through existing voltage-dependent channels. When the ionophore X537A was used, a selectivity series for divalent cations was $Ba^{2+} > Sr^{2+} > Ca^{2+} > Mn^{2+} \approx Co^{2+} > Ni^{2+} > Mg^{2+}$. However, the selectivity was similar to the affinity of the divalent cations for the ionophore (Kita and Van der Kloot, 1976). Black widow spider venom, which also acts as an ionophore, allowed several nonpermeant ions to increase spontaneous release, although the relative effectiveness was not determined (Misler and Hurlbut, 1979). The only response which is in qualitative variance with previous findings is that Mn decreased the m.e.p.p. frequency after toxin treatment. We might postulate that this is similar to the findings of Kita *et al.*, (1981), who found that at an elevated extracellular Mn concentration (10 m*M*), the m.e.p.p. frequency declined. They concluded that at high terminal levels, Mn may be inhibiting release, although at low levels, as we saw under control conditions (untreated, Table I) and others have seen (Balnave and Gage, 1973; Kita *et al.*, 1981), Mn increases spontaneous release. The potentiations of transmitter release by cobalt and nickel after toxin treatment reported here were of a high magnitude, but were seen at low concentrations of the divalent cations and without the need for nerve stimulation (TTX was always present) or nerve depolarization by potassium, and may reflect a more nonspecific permeability increase than seen by others.

Although it is clear that divalent cations can raise spontaneous release, how this occurs is still a question. One explanation is that divalent cations can bind to sites controlling release and act in place of calcium. If so, the relative effectiveness series would describe the relative affinities of the binding site involved in release. However, it is difficult at this time to separate the ion specificity of the toxin-induced permeability change from the ion specificity of the sites of action. In addition, divalent cations may be acting indirectly on transmitter release by producing a rise in internal calcium by either a displacement of sequestered calcium or a blockage of calcium uptake sites. Indicative of displacement due to competitive binding, divalent cations other than calcium were shown to precipitate in the same presynaptic locations as calcium by electron micrographic examination of frog neuromuscular junction (Ornberg and Reese, 1980; Politoff *et al.*, 1974). However, a recent study has shown a relatively lower affinity of the divalent cations, cobalt and nickel, for calcium binding sites in *Myxicola* axoplasm (Abercrombie *et al.*, 1980). The involvement of calcium transport in these experiments was suggested by the further increase in spontaneous release when β-BuTX-treated and cobalt-exposed muscles were returned to normal calcium-containing Ringer solution. The additional increase could be explained by a block by cobalt of calcium pumps or sequestering systems. Thus when calcium was reintroduced, intracellular calcium rose to a higher level than before cobalt exposure. Divalent cations have been shown elsewhere to inhibit calcium uptake into mitochondria (Carafoli and Crompton, 1977). In any case, divalent cations appear to be effective in potentiating transmitter release after permeability barriers have been broken down by a specific phospholipase treatment.

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